



Capillary zone electrophoresis of bacterial extracellular vesicles: A proof of concept



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ABSTRACT

The extracellular vesicles (EVs) released by plant pathogens of the *Pectobacterium* genus were investigated. The isolates were obtained using differential centrifugation followed by filtration and were characterized in terms of total protein content and particle size distribution. The transmission electron microscopy (TEM) analysis revealed the presence of two morphologically differentiated subpopulations of vesicles in the obtained isolates. The proteomic analysis using matrix-assisted laser desorption/ionization mass spectrometry with time of flight detector (MALDI-TOF/TOF-MS) enabled to identify 62 proteomic markers commonly found in EVs of Gram-negative rods from the *Enterobacteriaceae* family. Capillary electrophoresis (CE) was proposed as a novel tool for the characterization of EVs. The method allowed for automated and fast (<15 min per sample) separation of vesicles from macromolecular aggregates with low sample consumption (about 10 nL per analysis). The approach required simple background electrolyte (BGE) composed of 50 mM BTP and 75 mM glycine (pH 9.5) and standard UV detection. The report presents a new opportunity for quality control of samples containing EVs.

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1. Introduction

Extracellular vesicles (EVs) are supposed to be excreted by every living cell, which indicates their essential role in life processes [1]. In the case of Gram-negative bacteria, the vesicles are most frequently budded from the outer membrane of the cell, encapsulating the content of periplasmic space. Double-membrane EVs might transfer cytoplasmic proteins and nucleic acids acting as intercellular messengers, nutrient scavengers, and toxin transporters. EVs might also be implemented into the bacteriophage evasion

strategy of microorganisms or as a transfer medium of mobile genetic elements. EVs release is considered to be the most important feature in bacteria-host interplay, especially in pathogenicity [2,3].

Studying of bacterial and eukaryotic EVs carries similar difficulties concerning their characterization. Especially the estimation of purity and amount of EVs involves many problems and doubts [4]. Routinely performed simple measurements of total protein content might be biased by dissolved or aggregated proteins [4–6]. To face this problem, a ratio of particle number to total protein content was proposed [6] and is currently considered the most convenient way of EVs purity expression [4]. However, nanoparticle tracking analysis (NTA) or tunable resistive pulse sensing (TRPS), the techniques that are usually used for nanoparticles (NPs) counting, are not able to distinguish non-vesicular aggregates from EVs and are known to be operator dependent [4,5,7,8]. The utility of protein

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concentration measurement and particle number is also confounding for standardization of isolates containing various vesicles [4]. As a result, several alternative solutions for the estimation of purity and content of EVs in isolates have recently been proposed. Among the developed assays, measurements of lipid [9,10] or RNA [11] concentration should be mentioned. However, unlike proteins, lipids and nucleic acids might be found in isolates as non-vesicular impurities, which also creates a risk of significant bias of the assay. This is why more sophisticated, instrumental methods, like Raman spectroscopy [12] or flow cytometry [13–15], are currently of particular interest.

Capillary electrophoresis (CE) is an analytical technique used for high-performance separation of constituents according to their charge to size ratio, which makes CE applicable to a great variety of analytes, from small inorganic ions to small molecules (such as drugs), sugars, proteins, nucleic acids, and particles (NPs or even whole cells) [16–18]. The use of CE was also established in the pharmaceutical industry, especially for chiral analyses, purity assessment of active pharmaceutical ingredients, as well as quality control of manufactured antibodies and vaccines [19,20].

Bacteria of the *Pectobacterium* genus are Gram-negative peptolytic rods and common broad host range plant pathogens, presently classified into the *Pectobacteriaceae* family [21]. In 1992, a Japanese researcher Satoshi Fukuoka was first to observe production of vesicles by *Pectobacterium atrosepticum* [22]. Later, TEM images of the same microorganism with visible bubbles bulging from the outer membrane of the cell were published by Yaganza [23]. Since then, the topic of EVs release in *Pectobacterium* has not been continued, although a number of reports on the EVs significance for other plant pathogen virulence have been published (just to mention a few [24–26]).

In the presented work the EVs released by *Pectobacterium* sp. were investigated. The vesicles were characterized by TEM and DLS analyses. The proteome of *Pectobacterium* EVs was also characterized for the first time using mass spectrometry (MS). The achieved results indicate the role of EVs as a virulence factor in *Pectobacterium*. Moreover, a capillary zone electrophoresis (CZE) technique was proposed as a novel tool for the purity and content assessment of EVs in the analyzed isolates. It has been shown that CE is able to distinguish EVs from macromolecular aggregates. The developed assay is characterized by a relatively short time of single analysis (about 15 min) in a fully automated manner. The analysis consumes about 10 nL and requires as little as 5 μ L of sample which can be recovered for further experiments. Owing to the listed advantages, the CE is proposed as a candidate for routine analyses of EVs-containing samples.

2. Material and methods

2.1. Chemicals

Glycine, Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol), AMPSO (N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid), sodium dodecyl sulfate (SDS) and BIS-Tris propane (1,3-Bis[tris(hydroxymethyl)methylamino]propane; BTP) used in capillary electrophoresis experiments were purchased from Sigma (Steinheim, Germany). Sodium hydroxide was obtained from Avantor (Gliwice, Poland). All these chemicals were of analytical grade. In all the electrophoretic experiments deionized water produced with a Basic 5 system (Hydrolab, Wislina, Poland) was used.

2.2. Bacteria culturing and isolation procedure

Pectobacterium betavascularum strain IFB5271 (B5) was isolated from a sunflower of Mexican origin and obtained from

a collection of the Department of Biotechnology of the Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk [27]. *Pectobacterium* 9 M strain (=PCM2893 = DSM105717 = IFB9009) was isolated from Calla lily (*Zantedeschia* spp.) [28].

V-PEM medium used in experiments consisted of 0.32 g MgSO_4 , 1.08 g $(\text{NH}_4)_2\text{SO}_4$, 1.08 g K_2HPO_4 , 1.7 g sodium polypectate, 3 g meat peptone, and water adjusted to 1 L [29]. It should be borne in mind that water should be preheated to 100 °C. To avoid precipitation and clots the salts must be dissolved in 300 mL of water in order of their appearance in the recipe. The pH of the medium was adjusted to 7.2 and sterilized at 121 °C for 20 min.

Bacteria strains were kept in 40% glycerol (v/v) at –80 °C and cultivated on agar plates with a Mueller Hinton II agar or CVP medium [30]. Night culture was carried out in a liquid TSB medium at 28 °C with 120 rpm shaking. Then 220 mL of V-PEM medium was inoculated with 500 μ L of night culture and cultured for about 65 h at 28 °C with 120 rpm shaking. OD_{600} reaching 0.3 – 0.5 was taken as a good indication of culture condition and further isolation of EVs was carried out. Briefly, bacteria were centrifuged for 30 min at 4500 g, a supernatant containing OMV was proceeded to ultracentrifugation at 85,000 g (26 000 rpm; Beckman L-70 Ultracentrifuge; SW-28 Rotor) for 3 h 15 min at 4 °C. Debris containing OMV was suspended in 5 mL of cold 20 mM Tris/HCl (pH 7.6) or 20 mM Tris/100 mM AMPSO (pH 8.2) buffer. Samples were then filtered through PES filters with pores diameter of 0.22 μ m and stored at –20 °C.

We have submitted all relevant data from our experiments to the EV-TRACK knowledge base (EV-TRACK ID: EV190083) [31].

2.3. Capillary electrophoresis

All experiments were performed with the P/ACE MDQ plus system (Sciex, Framingham, MA, USA). The instrument was controlled with the 32 Karat software (version 10.2; Sciex). The separation process was conducted in uncoated fused silica capillaries (50 μ m i.d. x 30.2 cm total capillary length) using a constant voltage of 10 kV in normal polarity mode. The detection window was localized 20.2 cm from the capillary inlet. The capillaries were obtained from Polymicro Technologies (West Yorkshire, UK). Both sample chamber and capillary were thermostated during experiments at 25 °C. The injection of the sample was performed hydrodynamically (5 s, 3.45 kPa). The detection was performed at 200 and 230 nm. The wavelength of 200 nm was used for signals integration while 230 nm (considered as more selective) was used for peak identity confirmation.

The BGE was composed of 50 mM BTP and 75 mM glycine (pH 9.5). The solution was stored at room temperature and was stable for two weeks. All solutions used in CE experiments were filtered through a nylon syringe filter (0.22 μ m of pore diameter). The capillary rinsing was performed using a pressure of 69 kPa.

Every new capillary was rinsed with a 0.1 M NaOH aqueous solution (30 min) followed by water (10 min) and background electrolyte (BGE; 30 min). Subsequently, the capillary ends were dipped in vials filled with BGE and the conditioning was continued with an electric field (60 min, 10 kV).

At the beginning and end of every working day, the capillary was rinsed with 0.1 M NaOH and water (each solution for 10 min). Additionally, before the first analysis of the day the capillary was conditioned with BGE (30 min).

Between every run the capillary was flushed with BGE for 2 min. Next, a water dipping procedure was applied to prevent sample contamination with BGE [32]. During the water dipping procedure both ends of the capillary were placed in vials filled with deionized water which was immediately (the command 'Wait' was set at 0 min) followed by sample injection (5 s, 3.45 kPa) and

electrophoretic separation. The voltage was applied gradually for 0.5 min until 10 kV was reached. At the steady-state the electric current was constant and below 7 μ A throughout the whole separation process.

Corrected peaks area was used for CE data comparison with determined total protein content in isolates. Corrected peaks area was calculated with CE instrument operating software (32 Karat) using following formula:

$$A_{corr} = vA = \frac{L_d A}{t}$$

A_{corr} – corrected peak area; v – velocity of analyte migration; L_d – capillary length to detector; A – peak area; t – peak migration time.

The ratio of peak migration time to the migration time of EOF signal (corrected migration time) was used for data comparison [32].

2.4. Isotachopheresis

Isotachopheresis (ITP) experiments were performed with the P/ACE MDQ plus system using poly(DMA-GMA-MAPS)-modified capillaries (50 μ m x 30.2 cm). The leading electrolyte (LE) was composed of 15 mM Tris and 4.5 mM POPSO (pH 8.6) while the terminating electrolyte (TE) contained 20 mM Tris and 100 mM AMPPO (pH 8.0). The ITP experiments were performed under constant voltage at –20 kV using a semi-infinite injection mode. In this injection mode, the analytes are dispersed in TE and are continuously injected into the capillary filled with LE throughout the whole electrophoretic run. During analysis, the outlet of the capillary is dipped in the reservoir filled with LE. This type of injection in ITP was reported to provide the highest possible yield [33].

2.5. Capillary modification protocol

Bare fused silica capillaries of 50 μ m i.d. (Polymicro) were first rinsed with a 1 M NaOH solution (30 min) followed by rinsing with water (5 min), 0.1 M HCl solution (60 min) and again water (5 min). Such prepared capillary was flushed with a coating solution prepared by dissolving poly(DMA-GMA-MAPS) to a final concentration of 2% w/v in water and then diluting it 1:1 with a saturated (242 g/L) ammonium sulfate solution. This solution was flushed for 5 min and left filled for another 20 min. After this time the capillary was rinsed with water (5 min), dried with nitrogen and cured at 80 °C for 30 min. Afterward, the capillary was filled with BGE and stored at room temperature before use.

The detection windows in capillaries were burned before their modification to prevent polymer injury. The rinsing of the capillary with the above-mentioned solutions was performed at 0.3 MPa using the Nanobaume device (Western Fluids, Wildomar, CA, USA).

2.6. Total protein content

The total protein concentration was measured with the DC Protein Assay kit (Biorad, Hercules, CA, USA) using improved sensitivity protocol (20 μ L of sample was used) according to the manufacturer's recommendations. The samples were diluted with a 2% SDS in a 9 to 1 vol ratio before the assay [34]. The assay was performed in 96-well plates using the Infinite M200 plate reader (Tecan, Männedorf, Switzerland). The measurements presented in this paper were performed for filtered and non-filtered samples.

2.7. Dynamic light scattering

Nanoparticle size distribution was investigated using Litesizer 500 (Anton Paar, Graz, Austria). The measurements were done in

quartz cuvettes (standard or microvolumetric) at a measurement angle of 90°. The sample was thermostatted at 20 °C. The refractive index of the material and dispersant were set at 1.45 and 1.33, respectively. The viscosity was set at 0.001 mPa s. Each sample was measured in triplicate.

2.8. Transmission electron microscopy

The isolates (5 μ L) were deposited on the formvar support on copper mesh (200 mesh, Agar Scientific, Stansted, UK). After solvent evaporation the sample was contrasted with a 1% uranyl acetate and left for drying. The preparation was investigated with the use of the Tecnai G2 T12 Spirit BioTwin microscope (FEI Company, Hillsboro, OR, USA).

2.9. Proteomic analysis

The isolates were precipitated with acetonitrile (ACN) in a 1:4 ratio for 1 h at room temperature. The sample was centrifuged for 30 min at 16,000 g at 4 °C. The supernatant was discarded, and the residue was suspended in TLB buffer (0.1 M Tris – HCl, pH 8.0; 0.1 M dithiothreitol, 4% SDS) and thermostatted at 99 °C for 1 h. Next, samples were filtered and digested with trypsin followed by clean-up using ZipTip. 18 μ L of the digest was injected into the Acclaim PepMap 100 C18 column (75 μ m x 15 cm, 5 μ m, 100 Å, Thermo Fisher Scientific, USA) and fractionated at 7 °C in a reversed-phase mode using nano-LC (EASY-nLC IITM, Bruker Daltonics, Germany). The mobile phase was composed of (Eluent A) 0.05% aqueous solution of trifluoroacetic acid (TFA) and (Eluent B) 0.05% TFA in ACN and water mixture in a 9:1 ratio. Gradient elution was conducted using a 300 nL/min flow rate linearly increasing the gradient from 2 to 45% of Eluent B for 80 min. The fractions were deposited on the MTP AnchorChipTM 800/384 TF plate (Bruker Daltonics, Germany) by an automated system for fraction collection PROTEINEER fc II (Bruker Daltonics) followed by analysis with MALDI-TOF/TOF-MS ultrafleXtremeTM (Bruker Daltonics, Germany) equipped with a modified Nd:YAG laser (smartbeam IITM) operating at the wavelength of 355 nm and the frequency of 1 kHz. All mass spectra were generated by summing 500 laser shots. The spectra were recorded in the scan range of 680–4000 m/z in a positive ion mode. An acceleration voltage of 24.97 kV (IS1) was applied for a final acceleration of 22.37 kV (IS2). The LIFT voltages were set to 19.00 kV and 3.70 kV for LIFT1 and LIFT2. The identification of extracellular vesicles proteins was performed using BioTools (Bruker Daltonics, Germany) together with the MASCOT 2.4 in-house server (Matrix Science Ltd.) for searching against the *Pectobacterium* database (209,976 sequences; 73,432,114 residues; downloaded on 4 April 2019 from www.ncbi.nlm.nih.gov) with the following analysis parameters: mass accuracy of 50 ppm, mass tolerance of 0.3 Da, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine, deamidated and N-terminal acetylation as an allowable variable modification. Only the hits that were scored by the MASCOT software as significant ($p < 0.05$) were reported. The obtained results were examined in terms of the score level (greater than 90) and number of matched peptides (more than 2), which provided a >95% confidence level of protein identification [35,36].

3. Results and discussion

3.1. Characterization of EVs

The EVs isolation protocol included low-speed centrifugation of culturing medium for cell removal, ultracentrifugation of obtained supernatant for EVs sedimentation and filtration of re-suspended pellet through the PES filter (0.22 μ m of pore size). The TEM analysis confirmed the presence of cup-shaped vesicular structures that are typical of EVs (Fig. 1) [37]. Additionally, in the non-filtered

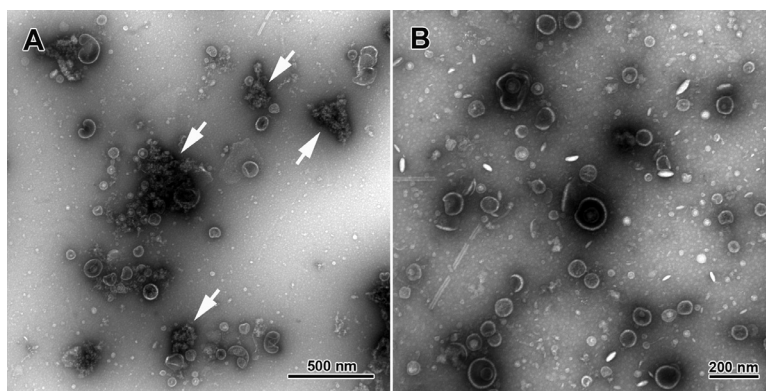


Fig. 1. Transmission electron microscopy photographs of (A) non-filtered and (B) filtered isolate. Wide-field images are provided in Supplementary Materials (Fig. 1S).

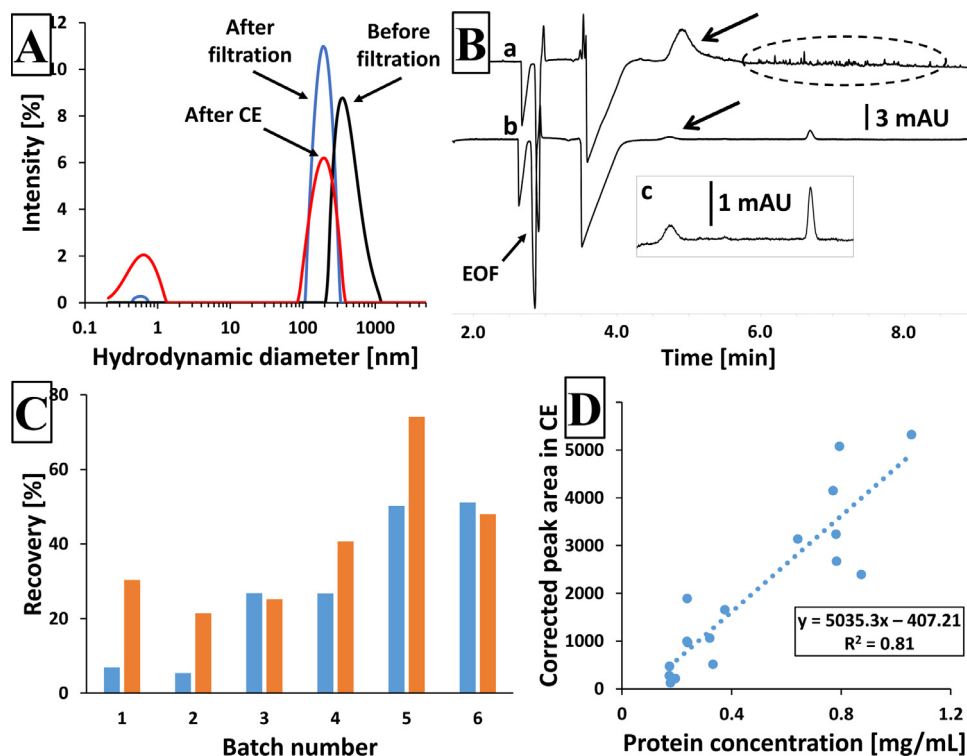


Fig. 2. The results of analyses of the isolates obtained from *Pectobacterium* sp. culturing media. (A) The DLS analysis of the isolate before and after filtration with a 0.22 μ m PES filter and after fractionation with CE. (B) The CZE analysis of the isolate sample (a) before and (b) after filtration with a 0.22 μ m PES filter. Figure (c) is a zoom of (b). Black arrows indicate the main signal generated by EVs. The black, dashed circle indicates macromolecular aggregates. Conditions: (BGE) 50 mM BTP, 75 mM Gly, pH 9.5; (voltage) 10 kV; (injection) 5 s, 3.45 kPa; (temperature) 25 $^{\circ}$ C; (detection wavelength) 200 nm. Total protein content in certain isolate was (a) 0.79 mg/mL and (b) 0.17 mg/mL. (C) A comparison of EVs recovery after filtration of the isolate estimated based on the (blue) CE analysis or (orange) total protein content measurement. (D) The correlation between total protein content in the filtered and non-filtered isolates and corrected peak area of the main signal recorded during their CE analyses ($n = 17$).

samples a number of undefined, macromolecular aggregates were found (indicated with arrows in Fig. 1A). The aggregates were not detected in the filtered samples (Fig. 1B).

The DLS analysis of isolates revealed the presence of microparticles (Fig. 2A). For analyzed non-filtered samples mean diameter (\pm Standard Deviation, SD) was 514 ± 133 nm. High SD value was attributed to the presence of various co-isolated components from bacterial culturing media. Interestingly, the filtration enabled to remove microparticles from isolates. The DLS analysis of the filtered samples determined the mean hydrodynamic diameter of EVs to be 184 ± 12 nm (\pm SD; Fig. 2A). It should be pointed out that particles smaller than 200 nm were not detected in the non-filtered isolates while this fraction of particles constitute the majority of NPs in the filtered samples. This was the result of masking of low intensity scattered light by bigger particles due to the polydispersity of sam-

ples (the polydispersity index of the non-filtered samples ranged from 22.6 to 30.6%) [38]. Nevertheless, the filtered isolates featured sufficient homogeneity for reliable DLS measurements (the average polydispersity index determined for 4 samples was 17.1 ± 3.2 (\pm SD)%).

The MALDI-TOF/TOF-MS analysis confirmed the presence of 62 proteins (Table S1), of which 18 (29% of total identified proteins) were outer membrane associated proteins and 2 (3% of total identified proteins) were periplasmic proteins. Outer membrane and periplasmic proteins are typically used for EVs identity confirmation [39]. Among these, common membranous markers such as OmpA, OmpF, and Lpp were detected. Most of the outer membrane and periplasmic proteins (>80%) were found to act as receptors or feature porin activity. The latter functionality was mainly assigned

to nutrients' transport through the membrane (KdgM, LamB, BtuB, FadL) and secretion (TssC, VipB, TagO).

More than 60% (38 proteins) of the identified proteins were cytoplasmic. Almost 70% of the cytosolic proteins identified in this study are involved in translation. Ten proteins (26% of cytosolic proteins) feature enzymatic activity and mainly take part in catabolic processes. The presence of cytosolic proteins in EVs isolates is often considered to be the result of sample contamination or inefficient purification [40–42]. While it might be the case, some attention should also be paid to the fact that these proteins are among the most frequently identified markers of EVs in Gram-negative bacteria [39]. Recently, Hong and coworkers showed the depletion of, inter alia, GroEL protein in *E. coli* EVs isolates after implementation of an additional purification step. Owing to this observation, GroEL was proposed to be used as an EVs purity marker. However, the stringent isolation protocol enabled only partial removal of this cytoplasmic protein [41]. Hong and coworkers' conclusions were in contradiction to the report of Joshi et al. where authors proved the insecticide role of EVs-transported GroEL protein [43]. According to the latter [43], the transport of cytoplasmic proteins in EVs has to undergo a defined mechanism. Later, the formation of double-layered, cytoplasm-carrying vesicles was shown to take place in Gram-negative bacteria [40,44]. Double-membrane EVs were found to be distinguishable from single-layered EVs using TEM microscopy. Indeed, TEM images of the isolates obtained in our study revealed the presence of vesicles with electron-dense content surrounded by a clear halo (Fig. 1B). According to the literature, such morphology is typical of double-layered EVs [40]. These vesicles were bigger and less abundant than single-layered EVs. The presence of double-membrane EVs explains the identification of cytoplasmic proteins in isolates.

3.2. Capillary electrophoresis of EVs

The size of most of EVs in investigated samples, determined with DLS and TEM analysis, was shown to be <200 nm. Due to this fact EVs in this study were considered as NPs. While NPs under electric field are highly vulnerable to aggregation as a result of particles collision, special attention should be paid during method development to minimize this threat [45]. Our group has recently shown that application of relatively big buffering counter-ions like BTP as BGE components sterically supports gold NPs stability during CE analysis [46,47]. For this purpose, the BGE used in current study was constructed with BTP and buffering co-ion (Gly) featuring pK_a value similar to BTP to achieve high buffering capacity.

The isolates obtained by ultracentrifugation were analyzed with CE without any further sample preparation. Standard hydrodynamic injection was performed (5 s, 3.45 kPa). The injection parameters were selected as a compromise between method sensitivity and separation efficiency. Loss of separation efficiency was proportional to the injection volume. Small conductivity difference between BGE (50 mM BTP/75 mM Gly; pH 9.5; conductivity \approx 0.1 S/m) and sample dispersant (20 mM Tris/100 mM AMPPO; pH 8.2; conductivity \approx 0.05 S/m) excluded stacking effect and contributed to the loss of separation efficiency almost proportional to the injection time in a range of 5 to 30 s (3.45 kPa).

Symmetrical signals featuring a relatively low separation efficiency ($N < 20\,000$ plates/m) were detected during CE of isolates (the exemplary signal was indicated with a black arrow in Fig. 2B (a)). Such signals are typically generated in CZE by dispersed NPs due to the particle size heterogeneity [46–50]. The assumption was made that the discussed signals in the electropherogram are due to the EVs presence in the assayed samples. Earlier migrating (mostly negative signals) species were mainly due to the buffering ions like AMPPO and Tris and were also detected during the analyses of blank samples. Next, a number of low intense, highly effi-

cient signals (often described in the literature as 'spikes' [46–50]) were detected, which was marked with a black, dashed circle in Fig. 2B (a). Interestingly, these signals were found not to feature defined electrophoretic mobility and their detection was random (sequential CE analyses of the same sample resulted in detection of various number of signals in the time range indicated with a black, dashed circle; Fig. 2B (a)). In the literature, the appearance of such 'spikes' during synthetic NPs analysis was linked with aggregation of particles [46–49,51,52], while Roberts and coworkers observed the same effect as a result of liposomes' destabilization [50]. In such cases, the UV detector response is not the result of light absorbance by solutes, but due to the light scattering on the detected objects [46,52]. Thus, the detection of spiky signals does not provide quantitative information on the amount of insoluble impurities in the sample. Moreover, irregular size and morphology of separated species lead to randomness of their detection during electrophoretic separation (undefined electrophoretic mobility). Indeed, the DLS analyses confirmed the presence of microparticles in the non-filtered isolates while the filtered samples were devoid of them (Fig. 2A). Macromolecular aggregates were also found in the non-filtered samples during TEM analysis (Fig. 1A). The CE analysis of the sample filtered through a PES filter (0.22 μ m of pore diameter) confirmed the identity of spiky signals in electropherograms, as they were not detected after filtration (Fig. 2B (b)).

It is possible to notice the reduction of the main signal area in the electropherogram of the filtered sample as compared to the non-filtered one (Fig. 2B). The loss of EVs during filtration is often reported in the literature and is typically estimated based on total protein content in samples [53–55]. In Fig. 2C the recovery was assessed with the use of the total protein content test and CE analyses. Only in the case of 2 out of 6 tested batches were the recovery values comparable, while in 4 other cases protein concentration measurements led to an overestimation of the EVs recovery after filtration. This might be explained by the presence of proteomic impurities in samples. For instance, in Batch 5 (Fig. 2C) the CE analysis of the filtered sample revealed the presence of spikes (macromolecular aggregates) which were expected to be removed by filtration. This might be explained by the filter membrane breakage or isolate contamination. In the case of the other three batches (Batches 1, 2 and 4) the bias was supposed to be caused by soluble impurities. Nevertheless, the presence of contaminants artificially increased the protein content in the filtered sample resulting in significant overestimation of EVs recovery. Linear correlation ($R^2 = 0.81$) between the corrected peaks area of the main signal in CE and the total protein content was found (Fig. 2D).

To confirm the identity of the main signal detected in electropherograms, the filtered isolates were fractionated with the CE system using ITP preconcentration with a semi-infinite injection mode (Figure S2). The composition of electrolytes (leading and terminating electrolytes – LE and TE, respectively) in ITP was adjusted to the electrophoretic mobility of EVs that enabled its selective preconcentration. For further improvement of the process yield, the fraction collection was repeated 7-fold for every assayed isolate. The protein content in the resulting fractions was below the detection limit of the protein assay kit used in this assay. The DLS analyses of resulting fractions confirmed the presence of particles featuring size distribution similar to those detected in initial isolates (Fig. 2A).

3.3. Discussion

The currently applied strategy for quality control of EVs isolates is based on the use of multiple techniques. While the quantification of total protein content and application of NTA feature some serious drawbacks (the issue was discussed in Introduction) [4–8], the development of a new strategy of isolate assessment seems

to be inevitable and vital for EVs sciences. The assessment of the quantity and purity of EVs in isolates is essential for their use not only in research, but also for treatment purposes [4,56]. In the last few years the EVs gained great interest as drug carriers and active ingredients in cancer and immunotherapy [57]. The medical use of EVs carries the need of rigorous control of their formulations and the present quality control methodology does not meet the applicable criteria [58]. These facts stand for rationale to implement separation techniques that are typically used in the pharmaceutical industry for quality control of active pharmaceutical ingredients and excipients.

The CE is an analytical technique with an established position in the pharmaceutical industry. Its application enables highly efficient separation and quantitation of compounds of interest and their impurities. The automation of the technique minimizes human error, improves the throughput and precision of the assay. The application of a commercial, analytical instrument is also advantageous in terms of inter-laboratory reproducibility. Moreover, the CE applicability in biological nanoparticles' analysis and vaccines' quality control has already been proven [17,20].

The work demonstrates the potential of the CZE in EVs isolates characterization. The undeniable advantages of CE include a relatively short time of a single analysis (<15 min), automation of the process, and negligible sample consumption (about 10 nL per assay). The quantification of vesicles with CE might be considered advantageous as compared to total protein content measurements, as well as particle counting techniques because the potential interferences are separated from vesicles' signal. At the same time, this feature enables to assess the purity of isolate, which is meaningful for biological experiments and especially important for pharmaceutical formulations.

The relationship between the corrected peak area in CE and protein content was found to be linear ($R^2 = 0.81$). The imperfect fit of the curve might be explained by the presence of co-isolated impurities, most likely soluble proteins. The negative value of intercept in the obtained linear regression equation ($y = 5035.3x - 407.21$) supports this explanation. Protein measurement test inaccuracy is also likely. The problem is well described in the literature [59]. Thus, future efforts should focus on the insightful quantitation of potential impurities. This can include the quantitation of soluble proteins concentration, application of particles counting methods as well as the use of few, various protein measurement kits. The development of a standard for method validation would also be favorable.

The sensitivity of the currently presented method was comparable to a commercial protein assay kit (DC Protein Assay kit, Bio-rad) and enabled the quantification of EVs in the samples featuring protein concentration down to 0.17 mg/mL. However, from our perspective there is a need to improve the sensitivity of the developed CE assay. Lower detection limits might be beneficial in screening tests when handling poorly purified samples. Although the coefficient of variation (CV) of the corrected peak areas for 6 consecutive runs at the limit of quantification (signal to noise 5) was satisfactory (<8.0%), greater sensitivity will also improve the precision of the method [60]. The repeatability of corrected migration times were < 1% for intra-day ($n = 6$) and inter-day ($n = 4$) assays. The reproducibility of the corrected peak area (total protein content: 0.33 mg/mL) assessed during four different days was also satisfactory (< 5%). Exemplary electropherograms presenting six subsequent runs were shown in Fig. 3.

A commonly encountered problem during CE of NPs [52,61], namely the adsorption of vesicles to the capillary wall, was not observed. Nevertheless, some attention should be paid to the physicochemical properties of the sample injected into a CE instrument. Significant differences between the viscosity and salinity of the analyzed sample and BGE might result in local disturbance of elec-

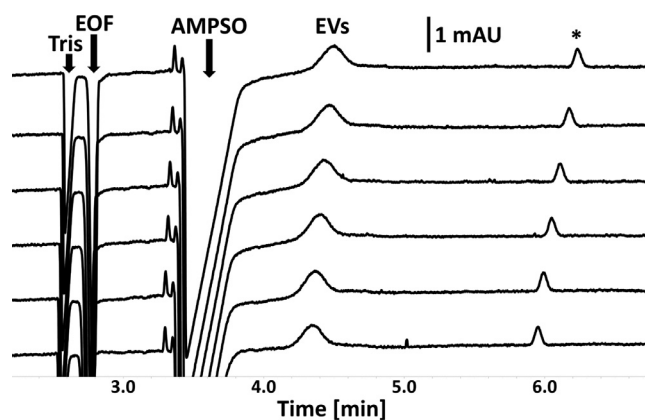


Fig. 3. Six consecutive runs of the same isolate using developed CE method. Conditions: (BGE) 50 mM BTP, 75 mM Gly, pH 9.5; (voltage) 10 kV; (injection) 5 s, 3.45 kPa; (temperature) 25 °C; (detection wavelength) 200 nm. The CV of corrected peak area and corrected migration times were 4.3 and 0.7%, respectively. Protein concentration determined for analyzed sample was 0.33 mg/mL. * - unknown signal.

trosmotic flow in the capillary. This, in turn, might lead to analysis disruption, decreased separation efficiency or peak tailing [32].

The electromigration phenomenon in the CZE enables the separation of species differing in charge to size ratio. The proper selection of BGE makes this technique capable of separating NPs by size [61]. Considering these fundamentals, CE is expected to distinguish EVs varying by size or surface charge. Despite both single- and double-layered vesicles were observed using TEM microscopy (Fig. 1B), we were not successful in separation these two subpopulations of EVs with CE.

Membrane and periplasmic proteins are typically reported as proteomic markers of Gram-negative bacteria EVs [39,41] and constituted a significant part of proteins (29%) identified in this study. The proteins, whose role is linked with membrane integrity (Lpp, Pal, TolB), might indicate their role in vesicles' release from cell membrane [62].

The detection of pectate lyases (Pel1 and Pel3) and oligoglycan transporter (KdgM) indicates the role of EVs in host-pathogen interplay [63]. While the detection of Pel lyases might be attributed to poor sample purity (these enzymes are known to be secreted), KdgM is an outer membrane transporting protein; hence, finding these three proteins might not be a coincidence. Pectate lyases are known to indirectly release plant response to bacterial infection [64]. Moreover, nutrient receptors and transporters are among the most frequently found proteins in bacterial EVs [39], and their presence was also confirmed in the assayed samples (fhuE, btuB, fadL, lamB). EVs might be used by bacteria as a nutrient scavenger and/or to shelter the enzymes from plant response until their delivery to host cells. The presence of adhesins (ompX, ompA) [39] and type VI secretion system components (tssc, vipB, tagO) is in agreement with this theory. Despite the fact that *Pectobacteriaceae* are considered to use the type II secretion system for lyases secretion [64], in another plant pathogen (*X. campestris*) the release of analogue enzymes using EVs was found to be independent of type II secretion system [25].

Interestingly, some proteins, which perform basic cellular functions, may play new roles in bacterial-environment relationships when released into the environment in vesicles. For instance, translation elongation factor Tu (TufB) in EVs produced by *X. campestris* enhances the immune response in attacked plants [65]. Chaperonin protein GroEL, secreted in EVs by *X. nematophila*, was found to feature a strong insecticidal effect on *Helicoverpa armigera* larvae [43]. It should be emphasized that both these proteins (TufB and GroEL) were identified in our study.

4. Conclusion

The CE offers some significant advantages for EVs characterization such as negligible sample consumption, automation of the assay and relatively short time of analysis (<15 min). While the validation protocol still needs to be developed, it seems that simple UV detection enables to quantify the amount of vesicles in isolates. What is more important, the CE allows to distinguish vesicles from macromolecular aggregates. It might be hypothesized that the application of more sensitive detection modes will enable to detect low abundant soluble impurities. We also expect that CE is able to separate various subtypes of EVs. These hypotheses will be investigated in our future work.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Martyna Piotrowska: Methodology, Investigation, Writing - original draft. **Krzysztof Ciura:** Writing - review & editing. **Michalina Zalewska:** Validation, Investigation, Writing - review & editing. **Marta Dawid:** Investigation. **Bruna Correia:** Investigation, Writing - review & editing. **Paulina Sawicka:** Investigation. **Bogdan Lewczuk:** Methodology, Investigation, Writing - review & editing. **Joanna Kasprzyk:** Methodology, Investigation, Writing - review & editing. **Laura Sola:** Methodology, Resources, Writing - review & editing. **Bartosz Wielgomas:** Methodology, Writing - review & editing. **Krzysztof Waleron:** Conceptualization, Methodology, Writing - review & editing. **Szymon Dziomba:** Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

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Supplementary materials

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